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Investigations into the Presence of a Competitive Inhibitor (Preprothrombin) in the Plasma of Chicks

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Attempts to verify the presence of a heat labile and on barium sulfate adsorbable substance postulated by other authors to inhibit the formation of prothrombin in dicumarol-induced hypoprothrombinemia in man have been carried out using dietetically vitamin K deficient and dicumarol poisoned chicks. The presence of such an inhibitor could not be confirmed under the experimental circumstances chosen.

The complex relationship between vitamin K and the indirect anticoagulants has received much attention within the past 25 years. The 4-hydroxycoumarin anticoagulants interfere indirectly with blood coagulation and produce a state that is similar but not necessarily identical with the simple alimentary vitamin K deficiency state.

According to generally accepted concepts, the vitamin K deficiency state, whether direct or induced by coumarin derivatives, involves lowering of the concentrations of prothrombin and certain other coagulation factors in the plasma.

This view has been elaborated further, by Hemker *et al.*[1] who have suggested the existence of a precursor of prothrombin tentatively called preprothrombin which is synthesized by the liver cells. The inhibitor is said to be heat labile, undialysable and adsorbable on to barium sulphate. It is converted into prothrombin (factor II) in a vitamin K dependent step under normal conditions, a reaction which is controlled by a feed-back mechanism that is dependent upon the concentration of prothrombin in the blood. If the amount of vitamin K is decreased or interfered with, this would result in a decreased amount of prothrombin and an excess amount of preprothrombin. The excess of the preprothrombin is shed into the blood stream and acts as a competitive inhibitor of prothrombin conversion giving rise to poor coagulation of the blood. Hemker's group used a modification of the Thrombotest for measuring

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the K-dependent procoagulants (factors II, VII, IX, and X) to demonstrate the presence of preprothrombin in the blood of elderly human patients on coumarin therapy.

The purpose of the present investigation is to determine if the competitive inhibitor is present in two samples of plasma obtained after different treatments from the same chick. The first sample was obtained when the chick was on a vitamin K-free diet. The second sample was analyzed when the chick was treated with the coumarin derivative coumachlor (3-*α*-*p*-chlorophenyl-*β*-acetylethyl)-4-hydroxycoumarin after recovery from the vitamin K-free diet. Chicks were used because of the facility of producing a K-deficient state in these animals. Since chickens lack factor IX, while still possessing the other three factors, the one stage prothrombin time was used to measure the clotting time of the chick plasma.

MATERIALS

Chicks (three weeks and day old) were obtained from Bellavista Farm, Roskilde, Denmark.

Thromboplastin extract was obtained from normal adult chicken brains that were mascerated after removal of the blood vessels, acetone extracted and dried. It was activated in Owren's buffer,[2] pH 7.35 at 37°C.

Sodium citrate: 3.8 % (w/v) solution in distilled water.

Calcium chloride: 0.02 M.

Barium sulphate: X-ray grade which was treated with 0.005 M trisodium citrate overnight, filtered and dried.

Isotonic saline.

Coumachlor: obtained from Danmarks Apotekerforenings Kontrollaboratorium.

Fibrinogen: human fibrinogen was isolated from Cohn fraction I (supplied by the State Serum Institute, Copenhagen) and purified by the method of Blombäck and Blombäck.[3]

Diet: Vitamin K-deficient diet was the same as reported by Dam and Søndergaard.[4] There were two control diets. One consisted of the K-free diet supplemented with Synkavite (Menadiol tetrasodium diphosphate), 1 mg/100 g of diet, and the other was a normal commercial diet supplied by A/S Karensmølle, Copenhagen.

Siliconized syringes, centrifuge tubes and pipettes were used in all the procedures for the preparation of the plasma.

METHODS

The chicks were made K-deficient by a diet lacking in this vitamin. Some of the chicks were cured by the administration of Synkavite and a normal diet for 2 weeks. After the two week curing period, the drug coumachlor was given to the chicks. The dose of the anticoagulant administered to the chicks ranged from 5 μg/g of body weight per day for one week treatment or given in doses of 30 μg and 20 μg/g of body weight at 44 and 20 h before withdrawal of the blood. In this way, it was possible to carry out simultaneous analysis of the plasma from the same chick under two different conditions, (1) as a result of a vitamin K-free diet and (2) as a result of anticoagulant treatment.

The blood was collected *via* the carotid arteries employing a sodium citrate solution (one part to nine parts of blood) to prevent clotting. The plasma was obtained by centrifugation in a refrigerated centrifuge. The blood and plasma were kept at 0°C except for carrying out the various treatments and prothrombin time determinations.

The one stage prothrombin time was employed to determine the coagulation velocity of the mixture. The coagulation mixture consisted of 0.1 ml of thromboplastin extract, 0.1 ml of 0.02 M $CaCl_2$ plus 0.1 ml of plasma sample (treated or untreated) or plasma that

had been diluted with isotonic saline. The enzyme systems and dilution (D) were made up in the same way as described by Hemker's group.[1] When D was equal to one, there was no dilution of the plasma, when D was equal to two, there was a 1:1 (v/v) dilution with saline. When D was equal to three, two volumes of saline were added and so on until maximum D value of nine was obtained. One enzyme system consisted of normal plasma with a constant amount of the vitamin K-deficient chick plasma. The second enzyme system was the same as the first system, except that the K-deficient chick plasma was either adsorbed or heat treated. It was not necessary to buffer the saline since the buffered thromboplastin extract and the plasma maintained the pH at 7.35.

In the adsorption experiment, $BaSO_4$ (100 mg/ml) was mixed gently with the given plasma at 37°C for 10 min. The adsorption mixture was then centrifuged twice at 20 000 *g* for 10 min to remove the adsorbent.

In the heat treatment experiment the plasma was heated to 50°C for 20 min and then cooled to 37°C.

In order to test the effect of the fibrinogen concentration in the plasma samples that were diluted with saline, a given amount of purified fibrinogen was added to the diluted samples to bring the concentration into normal ranges. The amount added was based upon the normal concentration of 400 mg%.

RESULTS AND DISCUSSION

The effect of the decreasing fibrinogen concentration in normal plasma by diluting with saline was not pronounced, as seen in Fig. 1. The addition of purified fibrinogen at higher D values did not lower the clotting time to the initial rate when D was equal to one. From this, it can be assumed that it is the lowering in the concentration of the other blood clotting factors, rather than the fibrinogen alone, that is mainly responsible for the prolongation of the prothrombin time.

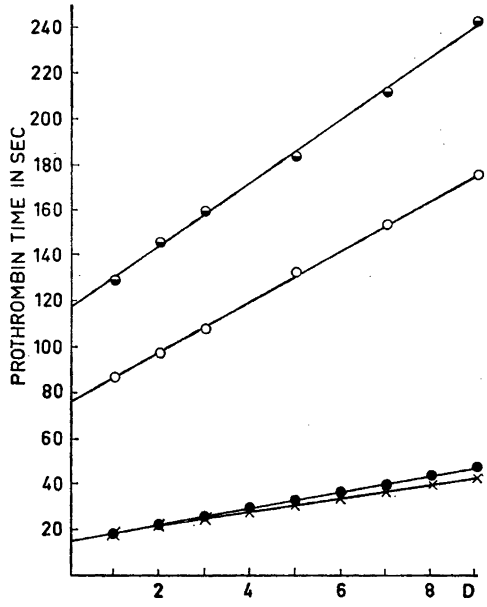

Fig 1. Effect of plasma dilution (D) *vs.* the clotting time (seconds). ● = normal plasma. × = normal plasma with added fibrinogen. O = plasma from the chick on a vitamin K-free diet. ◒ = plasma from the chick on coumachlor treatment.

Fig. 1 also shows the dilution results on the prothrombin time in the plasma from chicks treated with coumachlor and chicks that were on a vitamin K-free diet. Both plots had an elevated clotting time on extrapolation to infinite substrate concentration (t_m, following Hemker's terminology) as compared to chicks on a normal diet and on the vitamin K-free diet supplemented with Synkavite. The factor deficient plasma also showed greater slopes of the curves than the control plasma. This agrees with the Hemker group [1] who produced a relative (coumarin induced) vitamin K deficiency state in human patients. It was observed that the coumachlorized chick plasma did not always give a linear plot at increased dilution. It was also observed that the clot formation endpoint at increased dilutions was not as sharp in the coumachlorized chicks as it was in the chicks on the K-free diets. Since this could have an effect on the enzyme studies that were to follow in the plasma of the coumachlorized chicks, all future dilutions were made so that D was not greater than 5. It was also hoped that this step would control the parameter of fibrinogen concentration which should have a concentration of at least 80 mg%. A value below this level would slightly lengthen the prothrombin time, although it did not have a great effect on normal plasma when the results were plotted as in Fig. 1 (*i.e.* prothrombin time *versus* dilution).

Figs. 2A and 2B give the results of the enzymatic studies carried out with chick plasma. Barium sulphate treatment of the plasma from the chicks on

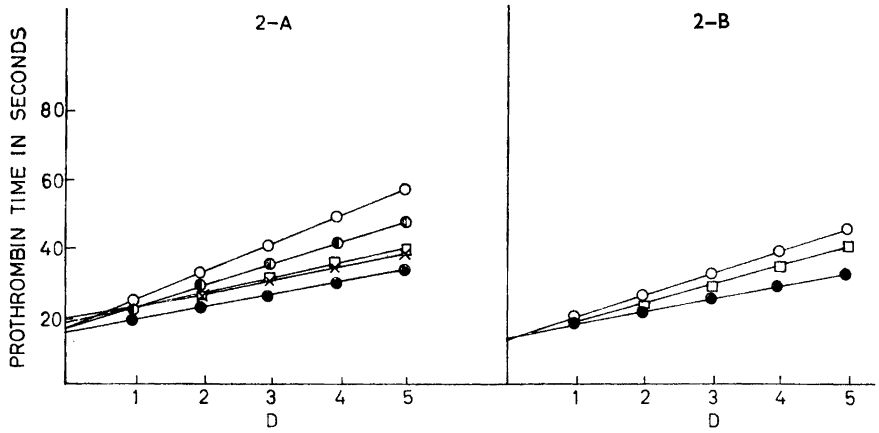

Fig. 2. Inhibitor studies: clotting time (seconds) *vs.* dilution (D) of the plasma mixture. ● = normal plasma. 2-A: Normal plasma with a constant amount of test plasma (0.005 ml nearly 1/20 of the mixture): ◐ = K-free, same line before and after adsorption. ○ = K-free diet, heat treatment. × = 44 h treatment with coumachlor without $BaSO_4$ treatment. □ = 44 h treatment with coumachlor with treatment on $BaSO_4$. 2-B: After one week's treatment with coumachlor: □ = before and after adsorption (one line). ○ = heat treatment.

a vitamin K-free diet or from the coumachlorized chicks failed to lower the respective lines to that of the control plasma, but yielded a plot that was almost similar to that of the plasma before treatment. These results were obtained despite the fact that the prothrombin time was much longer (greater than 15 min) in the adsorbed plasma. Any further loss of the vitamin K-dependent factors by adsorption was corrected by normal plasma in the enzyme system. Whether the animal was treated with the anticoagulant for two days or one week (Fig. 2B) made little difference on the experimental findings. Similar results were obtained with the heat-treated plasma except that these always had a greater slope. The heating process may have effected a partial destruction of factor V, the most heat sensitive coagulation component found in plasma. Due to the amount of plasma available, it was not possible to carry out factor V analysis. These observations are in contrast to those reported by Hemker *et al.*[1] Since the heating and adsorption treatments failed to decrease the lines of the two different plasmas to the control line of the normal plasma, it must be concluded that either (1) the inhibitor was not removed or (2), more likely, an inhibitor is not present at all.

The assumption that the coagulation defect induced by vitamin K deficiency and dicoumarol is due to the lowering of plasmatic coagulation factors without intervention of an inhibitor is supported by the recent work on prothrombin synthesis by Barnhart and coworkers at Detroit.[5-7] They used the fluorescent antibody technique of Coons and Kaplan [8] to evaluate factors II (prothrombin) and VII synthesis at the cellular level. Anderson and Barhart [9] correlated the cellular hepatic fluorescence (found in liver sections) with the circulatory coagulation factors during the alteration of synthesis by dicoumarol. They found that the parenchymal cells of the liver could increase prothrombin (and factor VII, if this protein possessed similar determinant groups) from 10 to 90 % fluorescence in 4—5 h following vitamin K_1 administration in dicoumarolized dogs, whereas it took only 1—2 h in dogs without dicoumarol treatment. The dicoumarolized dogs had an almost simultaneous lowering of their prothrombin times to normal values. The demonstration of decreased prothrombin (and factor VII) in the liver and blood of dogs on dicoumarol treatment by the Detroit group and the results of the present enzyme studies prove that vitamin K deficiency must interfere with the hepatic synthesis of the blood clotting factors.

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